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Sex identification in *Nepenthes adrianii* from Baturraden Botanical Garden: Genetic analysis using RAPD Markers

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ABSTRACT. *Nepenthes adrianii* is one of the pitcher plant species that grows endemically in Mount Slamet, Central Java. At present, it is one of the pitcher plant collections of Baturraden Botanical Garden. Since *N. adrianii* is dioecious and both sexes are difficult to distinguish morphologically, early sex determination supporting its conservation at Baturraden Botanical Garden is needed. One approach is performed by utilization of RAPD molecular markers. Hence, this study aims to know whether differences in RAPD patterns between male and female *N. adrianii* exist and to determine the differences. Genomic DNA were extracted from leaves of four males, two females, and two sexually unidentified individuals. The extracted DNA were then used to analyze DNA variation between male and female *N. adrianii* employing the RAPD technique. As many as five oligonucleotide primers (OPA-15, OPK-16, OPP-15, OPP-08, and OPO-08) amplify *N. adrianii* DNA. The results showed that one primer, i.e., OPK-16 (5′-GAGCGTCGAA-3′), produces a specific band of approximately 290 bp, which is only found in female plants. This band is assumed to be related to gene(s) controlling sex determination in *N. adrianii*. Young *N. adrianii* seedlings can use RAPD marker for sex determination.

Keywords: *Nepenthes adrianii*; oligonucleotide primers; RAPD; sex identification

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INTRODUCTION

Nepenthes adrianii is a pitcher plant species belonging to rare and nearly extinct groups of species listed in appendices I and II of the Convention on International Trade of Endangered Species (CITES) (Batoro & Wartono, 2017). In addition, to be endemic in Mount Slamet, Central Java, *N. adrianii* is nowadays one of the floral collections of Baturraden Botanical Garden, located on the southern slope of the mountain. It is an ex-situ conservation region for various plant species covering 143.5 ha area with altitudes of 600 to 800 m above sea level, intended mainly to preserve characteristic plant species from mountain areas in Java Island (Mandiriati *et al*., 2017).

To support ex-situ conservation of *N. adrianii* in Baturraden Botanical Garden, early detection of sex in the plant species is needed. *N. adrianii* is dioecious or possessing male and female flowers separated in different individuals. In their natural habitat, male *N. adrianii* individuals are much more easily found than females, as is the case in most other pitcher plant species (Adam *et al*., 2011; Sweat & Bodri, 2014; Handayani, 2017). Meanwhile, sex determination in dioecious plants is crucial, mainly when each sex will be developed as individuals of economic significance, and distinguishing sex at an early stage of development is considerably challenging to do (Zarek, 2018).

One approach to early identification of sex in *N. adrianii* can be performed using DNA analysis employing particular molecular markers, e.g., Random Amplified Polymorphic DNA (RAPD) markers. The RAPD technique is widely known as a simple and relatively inexpensive approach to applying. It involves amplifying many specific sites along genomic DNA complementary with short random primers of about ten nucleotides, resulting in amplicons of different sizes (Kumar & Gurusubramanian, 2011; Abdullah *et al*., 2019). In addition to its simplicity and

cheapness, this technique shows some other advantages that the primers used are universal and applied in either prokaryotic or eukaryotic species (Mohkam *et al*., 2016; Johnson-Mackinnon *et al*., 2019). As well, it does not require any information on the target sequence. Despite a relatively low reproducibility, the RAPD technique can rapidly detect polymorphism in several loci (Şahin-Çevik & Moore, 2012; Pandin, 2015).

This study aims to identify the presence of different RAPD patterns between male and female *N. adrianii* and examine what the differences are. By finding genetic marker(s) determining sex in *N. adrianii*, developing the respective sex corresponding to its specific utilization since the plant is still in the seedling phase.

MATERIALS AND METHODS

An explorative method employing the purposive random sampling technique was applied in this study regarding plant sampling. All plant samples were taken randomly from the collections of Baturraden Botanical Garden, including four males, two females, and two sexually unidentified individuals. Molecular analysis was performed in the Laboratory of Genetics and Molecular Biology, Faculty of Biology, Universitas Jenderal Soedirman.

Genomic DNA extraction. Genomic DNAs were extracted from the thoroughly expanded leaves following CTAB method (Abdel-Latif & Osman, 2017). Individual leaf of 0.1 g was cut into small pieces and put into a 1.5 ml microtube. Then, 800 µL CTAB buffer previously heated at 65°C for 30 mins was added. The leaf pieces were crushed and powdered by using a mini-beadbeater for four mins. Afterward, the sample was put into a waterbath of 65°C for 60 mins, in which the microtube was turned upside down in every 10 mins. The sample was then taken from the water bath and allowed to cool down at room temperature for two mins, after which 500 µL chloroform-isoamyl alcohol (CIAA) was added. It was mixed gently and vortexed for five mins. The mixture was then centrifuged at 12000 rpm for 15 mins. The supernatant was moved carefully into a new microtube. A total of 3 M sodium acetate of 1/10 supernatant volume was added and mixed gently. Cold isopropanol of 2/3 total volume (sodium acetate plus supernatant) was then added to the mixture and mixed gently by flipping the tube. This mixture was then kept in the freezer for 24 hours. The sample was centrifuged at 12000 rpm for 10 mins, the supernatant was discarded, and the DNA pellet was washed with 500 µL ethanol 70% by flipping the tube. The mixture was centrifuged again at 12000 rpm for five mins, the supernatant was discarded, and the DNA pellet was air-dried. The DNA pellet was then dissolved into 100 µL TE buffer and kept at 4oC before quantification was performed by using GeneQuant.

Amplification of RAPD markers. Five random primers were previously reported to distinguish sex in some plant species, i.e., OPA-15, OPP-15. OPO-08, OPP-08, and OPK-16 were used to amplify RAPD markers in this study. The primers are listed in Table 1.

Table 1. Random primers used in this study.

No.	Primer	Sequence (5'- 3')	Usage	Reference
1	OPA-15	TTCCGAACCC	marker for male *Nepenthes mirabilis*	Mokkamul *et al*. (2007)
2	OPP-15	GGAAGCCAAC	marker for female *Nepenthes mirabilis*	Enjelina *et al*. (2018)
3	OPO-08	CCTCCAGTGT	marker for male *Trichosanthes dioica*	Kumar *et al*. (2008)
4	OPP-08	ACATCGCCCA	marker for male *Salacca zalacca*	Parjanto *et al*. (2006)
5	OPK-16	GAGCGTCGAA	marker for male *Nepenthes ampullaria*	Enjelina *et al*. (2018)

The PCR-RAPD was carried out in a total volume of 12 µL consisting of 2 µL (10 ng) template DNA, 0.75 µL (10 pmol) random primer, 5 µL (1 unit) Kappa mix, and 4.25 µL nuclease-free water. Amplification was performed using PCR PTC-100 Programmable Thermal Cycler machine in the following condition: pre-denaturation at 95°C for 3 mins; 40 reaction cycles consisting of denaturation at 95°C for 1 min, annealing at 35°C for 2 mins and extension at 72°C for 2 mins; final extension at 72°C for 10 mins. The PCR

products were resolved in a 2% agarose gel electrophoresis using TBE buffer 1x. After being stained with ethidium bromide, the gel was exposed to UV transilluminator for visualization.

Data Analysis. Data on PCR bands (Table 2) were analyzed descriptively according to their occurrence in the respective sex. Only one of the sexes can be used to distinguish between male and female *N. adrianii*.

Table 2. Polymorphism of PCR-RAPD bands in *Nepenthes adrianii* from Baturraden Botanical Garden.

Primer	Number of monomorphic bands	Number of polymorphic bands	Total
OPA-15	6	4	10
OPP-15	5	4	9
OPO-08	1	6	7
OPP-08	3	8	11
OPK-16	2	6	8
Total	17	28	45
	37.7%	62.3%	100%

RESULTS AND DISCUSSION

Amplification of RAPD markers using primer OPA-15 results in 66 PCR bands, creating ten band patterns, six of which are monomorphic and the other four are polymorphic. All samples show amplicons with primer OPA-15, but no specific band distinguishing between male and female individuals is produced (Fig. 1). A similar result was reported in *N. gymnamphora*, where OPA-15 could not produce any PCR band distinguishing sex (Pertiwi *et al.*, 2019). Oppositely, OPA-15 had successfully produced a DNA fragment of 750 bp distinguishing sex in both *N. gracilis* and *N. mirabilis* (Mokkamul *et al*., 2007). It indicates that primer capable of distinguishing sex in *N. gracilis* and *N. mirabilis* could not automatically be used in other *Nepenthes* species such as in *N. adrianii* and *N. gymnamphora*. Meanwhile, an RAPD marker of 1625 bp amplified using OPA-15 specifically observed in male *Momordica dioica* was obtained (Baratakke & Patil, 2009).

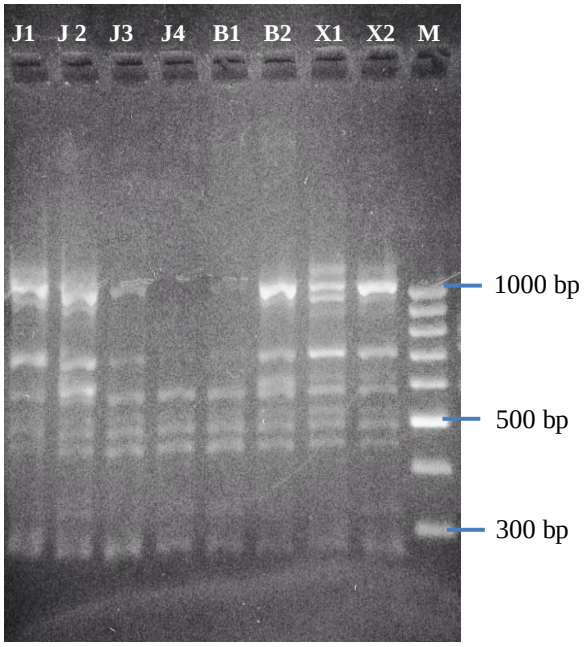

Fig. 1. PCR-RAPD using primer OPA-15 (M= DNA Ladder; J1= male 1; J2= male 2; J3= male 3; J4= male 4; B1= female 1; B2= female 2; X1= unknown sex 1; X2= unknown sex 2).

As many as 57 PCR bands belonging to nine patterns are amplified using primer OPP-15, five of which are monomorphic, and the remaining four are polymorphic. Nevertheless, no specific band distinguishing between male and female *N. adrianii* is produced (Fig. 2).

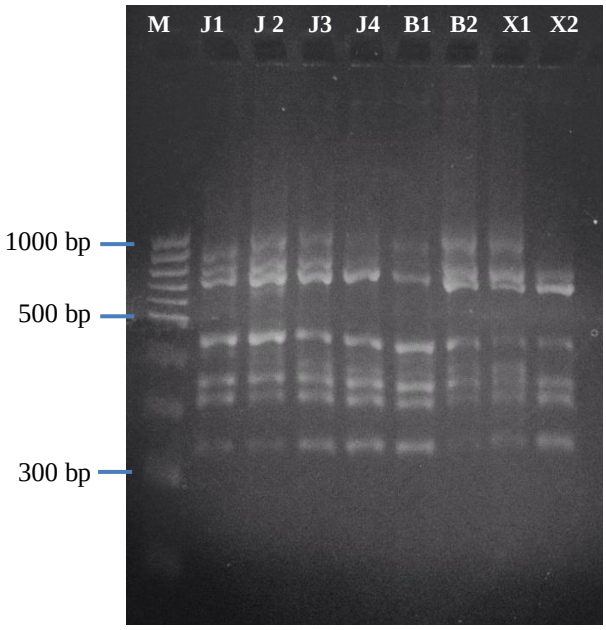

Fig. 2. PCR-RAPD using primer OPP-15 (M= DNA Ladder; J1= male 1; J2= male 2; J3= male 3; J4= male 4; B1= female 1; B2= female 2; X1= unknown sex 1; X2= unknown sex 2).

In *N. mirabilis*, however, primer OPP-15 had successfully amplified a DNA fragment of 650 bp found merely in female individuals (Enjelina *et al*., 2018). The PCR-RAPD using primer OPO-08 results in 33 bands with seven band patterns, none explicitly distinguishing between male and female *N. adrianii*. Two groups of bands, i.e., one monomorphic and six polymorphic bands, are observed (Fig. 3). Conversely, primer OPO-08 had been proved to amplify RAPD marker of 1263 bp, which was only observed in male *Pandanus fascicularis* (Vinod *et al.*, 2007). As well, this primer had successfully produced a DNA fragment of 350 bp specifically found in male *Trichosanthes dioica* (Kumar *et al.*, 2008). Hence, these are different from the case in *N. adrianii*, where primer OPO-08 produces no specific band distinguishing sex.

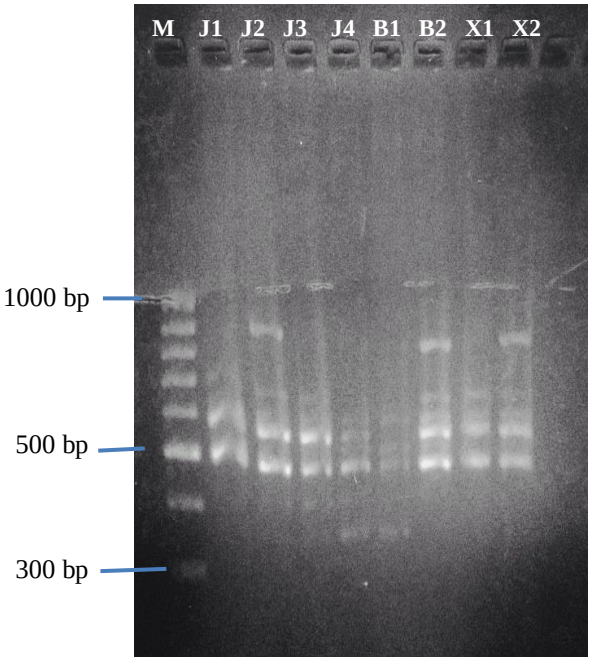

Fig. 3. PCR-RAPD using primer OPO-08 (M= DNA Ladder; J1= male 1; J2= male 2; J3= male 3; J4= male 4; B1= female 1; B2= female 2; X1= unknown sex 1; X2= unknown sex 2).

Amplification using primer OPP-08 results in 63 PCR bands with 11 band patterns. No specific band distinguishing sex in *N. adrianii* is observed despite three monomorphic and eight polymorphic bands (Fig.4). It contrasts with was reported in *N. gymnamphora*, where OPP-08 had successfully amplified an RAPD marker of 250 bp specifically observed in female individuals and 300 bp that was only present in male individuals (Pertiwi *et al*., 2019). Similarly, an RAPD marker of 400 bp was found only in male *Salacca zalacca* when amplified using primer OPP-08 (Parjanto *et al*., 2006).

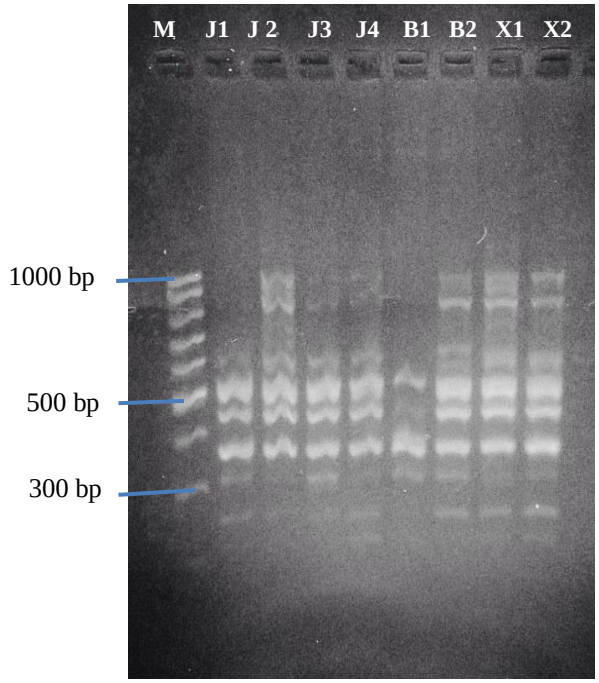

Fig. 4. PCR-RAPD using primer OPP-08 (M= DNA Ladder; J1= male 1; J2= male 2; J3= male 3; J4= male 4; B1= female 1; B2= female 2; X1= unknown sex 1; X2= unknown sex 2).

By the use of primer OPK-16, a total of 48 PCR bands belonging to eight band patterns are produced. It is also shown that two monomorphic and six polymorphic bands are observed (Fig. 5). The monomorphic bands are those of 420 bp and 580 bp in length. Unlike the other four primers, OPK-16 results in an amplicon of 290 bp that can distinguish sex in *N. adrianii* since it is only found in female samples. Hence, this PCR band is presumably related to genes controlling sex in *N. adrianii*. Correspondingly, primer OPK-16 had successfully amplified RAPD markers distinguishing sex in *N. ampullaria* because those of 400 bp, 500 bp, and 850 bp were specifically observed in only male individuals (Enjelina *et al*., 2018). In addition, it can be seen in Fig. 5 that amplicons of 290 bp are also present in both samples X1 and X2, which are

sexually unknown. Therefore, it is strongly assumed that they are female individuals.

Analysis of locus polymorphism of the eight *N. adrianii* samples using all the five primers in this study reveals that 28 polymorphic bands (62.3%) and 17 monomorphic bands (37.7%) of a total of 45 PCR bands are produced. On average, six polymorphic bands per primer are produced. The data on locus polymorphism are summarized in Table 2. The presence of a polymorphic band indicates genetic variation among individual samples, though this is not necessarily related to sex differences among samples (Rameshkumar *et al*., 2019).

A previous study on RAPD profiles in various pitcher plants from Baturraden Botanical Garden resulted in PCR bands ranging from 130 to 1,500 bp. The random primers used were ES10G23, ES10A26, ES10C24, ES10G33, OPA-2, OPA-9, OPA-13, OPB-3, OPB-5, and OPB-7 (Mayangsari *et al*., 2017). Different amplification products might result from the distribution of base sequences along the genome complementary to the respective primer. Differences in the distance among primer annealing sites would give rise to amplicons of various sizes (Gusmiaty *et al.*, 2012).

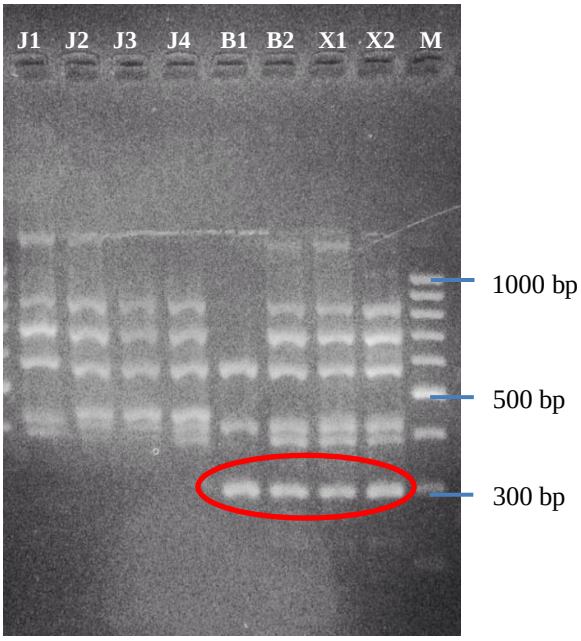

Fig. 5. PCR-RAPD using primer OPK-16 (M= DNA Ladder; J1= male 1; J2= male 2; J3= male 3; J4= male 4; B1= female 1; B2= female 2; X1= unknown sex 1; X2= unknown sex 2).

RAPD marker of 290 bp amplified with primer OPK-16 enables sex determination in *N. adrianii* at an early stage of development, such as at the seedling phase. RAPD is not affected by plant individuals' environmental conditions and growth phase as with other molecular markers. It is applied in plant sex determination because different occurrences in both sexes exist (Probojati *et al*., 2019). Besides, using RAPD markers to distinguish sex in dioecious plant species has some advantages compared to that of other molecular markers such as RFLP. RAPD technique is relatively simple to apply, cheap, needs fewer samples and usually results in high polymorphism (Vaidya & Naik, 2014).

Along with inter-simple sequence repeat (ISSR), RAPD markers have been the most widely used molecular markers in sex identification in dioecious plant species (Ghumatkar *et al*., 2015). For instance, 14 random primers were used to identify sex in five *Carica papaya* varieties, two of which could determine sex types of the varieties (Prihatini *et al.*, 2019).

Unlike in the animal kingdom, in which sexual dimorphism is widespread, only about 6% of plant species show such a sexual system. Most flowering plants are monoecious or even hermaphroditic, where both male and female reproductive organs exist in the same flower. On the contrary, sex types in dioecious plant species are determined mainly by the sex chromosome system, which can be either male heterogamety (male XY, female XX) or female heterogamety (male ZZ, female ZW). In male heterogamety, the fully sex-limited region is transmitted via males, while in female heterogamety, it is performed through females. Moreover, sex chromosomes in plants can be classified into two types, i.e., homomorphic, which is cytologically indistinguishable from autosomes, and heteromorphic, which is different from autosomes (Mintah, 2018).

The presence of sex chromosomes in *Nepenthes* has been reported as homomorphic types. It was found that some loci occurred only in males and the XY sex chromosome system seemed to exist. One gene located on the Y chromosome was strongly assumed to be responsible for pollen

development and thus could be used as a genetic marker in molecular sexing at the vegetative phase (Scharmann *et al.*, 2019).

Since a PCR-RAPD band for the female marker in *N. adrianii* has been obtained using primer OPK-16, it can also create a more stable and reliable marker. It is despite the simplicity of RAPD markers, some limitations, particularly concerning reproducibility, exist. Sequence characterized amplified region (SCAR) markers, which are more reproducible and specific than RAPD markers, should be constructed by cloning and sequencing the amplified band (Zhou *et al*., 2018).

CONCLUSION

Of all random primers used in this study, only OPK-16 produces a PCR-RAPD band of about 290 bp that serves potentially as female marker in *Nepenthes adrianii*. It can be employed to design a SCAR primer for molecular sexing in the species so that sex determination can be performed even when the plant is still in the vegetative phase.

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